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Direct Interaction Between Autonomic Nerves and the Immune System

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This project examines interactions between autonomic nerves and the immune system. Noradrenergic sympathetic nerves are present in spleen and lymph nodes, particularly in T cell and macrophage compartments, shown by light and EM immunocytochemistry during the past year. Some nerve terminals form synaptic-like contacts with T lymphocytes in spleni white pulp. Neurochemical studies have shown release and availability of micromolar concentrations of norepinephrine, and an absence of acetylcholine or choline acetyltransferase in spleen, suggesting only noradrenergic and not cholinergic innervation. Following denervation of noradrenergic nerves to spleen and lymph nodes, many immune parameters are altered, including 1° and 2° antibody responses, mitogen responses, delayed-type hypersensitivity responses, B lymphocyte proliferation, cytotoxic T cell activity, and NK cell activity. Cold exposure also can lead to altered immune responses. These studies indicate thet the noradrenergic nerves innervating spleen and lymph nodes are necessary for immunocompetence and that norepinephrine exerts an immunomodulatory influence.

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I. INTRODUCTION

This ongoing project continues to examine the interactions between the autonomic nervous system and the immune system in young adult male C3H and BALB/c mice and young adult rats. Evidence from our laboratory and others has revealed direct autonomic innervation of the parenchyma of lymhpoid organs by noradrenergic nerves, with a particular concentration of terminals in regions where T lymphocytes and macrophages are found, and has further demonstrated changes in antibody and cell-mediated immune responses following alterations of the noradrenergic nerve supply to spleen and lymph nodes. We hypothesized that the autonomic nerves supplying lymphoid organs represents an important mechanism through which aversive environmental conditions may lead to altered immune functions. These autonomic nerves and their transmitters in spleen and lymph nodes are being manipulated experimentally by:

(1) Surgical denervation, including coeliac/superior mesenteric ganglionectomy to remove the noradrenergic sympathetic innervation. This source of innervation has been established by tracing studies from our laboratory.

(2) Pharmacologic manipulation to alter the noradrenergic innervation, including 6-hydroxydopamine (6-OHDA) for chemical sympathectomy, tricyclic reuptake blockers for NE enhancement, propranolol for beta-adrenergic blockade, and phentolamine for alpha-adrenergic blockade. Cholinergic agents were not examined due to the findings of our anatomical and neurochemical studies revealing that cholinergic nerves to not innervate secondary lymphoid organs.

(3) Mild or extended cold exposure, known to enhance peripheral sympathetic turnover of norepinephrine.

Following these experimental manipulations, we have examined both neural and immune parameters. For investigations of autonomic innervation of the spleen and lymph nodes, we have undertaken light and electron microscopic analysis of noradrenergic (tyrosine hydroxylase positive) terminals and their relationship to lymphocytes and macrophages, using histochemical and immunohistochemical markers for the transmitters and the subsets of T lymphocytes (helper and suppressor), B lymphocytes, and antigen-presenting (ED-3) macrophages, with subsequent examination with quantitative light microscopy of double-labelled sections and electron energy loss spectroscopy at the ultrastructural level. We also have carried out neurochemical analysis with LCEC (high performance liquid chromatography with electrochemical detection) of NE and its metabolites in specific zones or micropunched regions of the spleen and of single lymph nodes for a comparison with anatomical sections. We have utilized in vivo dialysis to obtain an assessment of more dynamic measures of NE release and availability in the splenic microenvironment.

For immunological assessment, we have examined cell-mediated immune reactivity to contact sensitizine agents and to cell surface antigens, proliferative responses of lymphocytes, natural killer (NK) cell function, cytotoxic T cell function, and have begun to examine natural resistance and acquired immunity to herpes simplex virus type 1.

This combined effort seeks to elucidate the means by which the autonomic nervous system and the immune system interact physiologically and under aversive environmental conditions that may undermine the health and functional capabilities of military personnel. We consider the investigation of basic mechanisms and principles underlying neural-immune communication to be the best approach to this end. We continue to seek to define strategies by which immune competence can be maintained optimally in the presence of such aversive environmental conditions. This objective may best be met by controlling the

system that we consider to be an important modulator of immune function, the autonomic nervous system.

II. PROGRESS REPORT

A. Innervation Studies

1. Splenic Innervation

Fluorescence histochemical studies from our laboratory (Williams et al., 1981; Felten et al., 1985) demonstrated abundant NA fibers branching from the vasculature in the white pulp and ending in the parenchyma. Our recent use of immunocytochemical localization of tyrosine hydroxylase (TH), the rate-limiting enzyme in NE synthesis, and a specific marker of catecholaminergic nerves, has permitted more precise determination of the specific compartmentation of NA fibers, and has confirmed the following pattern of innervation (Figs. 5-7) (Ackerman et al., 1987; D. Felten et al., 1987a).

NA fibers in rodents arising from the superior mesenteric-coeliac ganglion (D. Felten et al., 1986) traveled with the splenic artery as the splenic nerve, which contains 97% NA postganglionic sympathetic axons (Elfvin, 1961), and entered the hilar region of the spleen. The fibers then either traveled with the vasculature towards the central arteriolar system of the white pulp, or densely innervated the trabecular/capsular system; only rarely were nerve fibers seen in the red pulp, constituting less than 1% of the NA innervation. The TH^+ fibers formed plexuses around the central artery and its branches in the white pulp. Some fibers remained close to the smooth muscle of the arterioles, while others ran alongside the arterioles several cell layers deep in the PALS. Additional fibers radiated from the central arterioles into the parenchyma of the PALS, rich in T lymphocytes ($OX19^+$)(Dallman et al., 1984). These fibers were adjacent to both $W3/25^+$ (MHC class II-restricted T "helper") cells and $OX8^+$ (MHC Class I-restricted T "cytotoxic/suppressor") cells (Barclay, 1981) which were tightly intermixed in the PALS. Additional linear arrays of fibers or individual TH^+ fibers coursed along the marginal sinus, adjacent to $ED3^+$ macrophages. Some fibers extended beyond the marginal sinus into the outer marginal zone. Other long nerve fibers coursed along the parafollicular zones on both the inner and outer surfaces. Only infrequently were small nerve fibers seen within a follicle, and even then were associated with small clusters of T lymphocytes. Zones of surface IgM^+ B lymphocytes within the follicles were not innervated except at the outermost parafollicular regions. Double staining for the TH^+ nerve fibers and lymphocyte or macrophage markers confirmed that the nerve fibers distributed mainly into the T lymphocyte compartment of the PALS, the macrophage-rich area of the marginal sinus, and the parafollicular zones.

Electron microscopic immunocytochemical studies were carried out to establish the existence of contacts between the TH^+ nerve fibers and parenchymal cells in the PALS. (S. Felten and Olschowka, 1987; S. Felten et al., 1987). The electron microscopic observations confirmed the light microscopic immunocytochemical findings, and revealed contacts made by the NA terminals. Nerve terminals were positively identified by the diaminobenzidine reaction, their size and appearance, and contacts with target cells. TH^+ terminals were found between the media and adventitia of the central arterioles, always separated from the smooth muscle cells by a basement membrane, suggesting that what have long been considered "classical" synapses, with nerves directly contacting blood vessels, may actually be paracrine interactions.

However, the TH^+ terminals directly contacted lymphocytes in the PALS, both adjacent to the smooth muscle layer and deeper in the PALS, distant from the arterioles. These terminals revealed long smooth zones of contact with the lymphocyte plasma membrane, separated by only 6 nm. (Gap junctions are 2 nm, classical synapses are 20 nm.) The apposing membranes often were prominent or slightly thickened. In many cases, the TH^+ terminal indented into a lymphocyte. Similar tight contacts were found in the marginal zone between TH^+ terminals and lymphocytes. In addition, some terminals

were found adjacent to macrophage-like cells or processes. No post-synaptic specializations were identified; however, such CNS-type synaptic specializations are seldom found in peripheral sympathetic NA fibers and their target cells.

Over the past 25 years, scattered reports of electron microscopic observations in the spleen (Galindo and Imaeda, 1962; Moore et al., 1964; Zetterstrom et al., 1973; Reilly et al., 1979; Blue and Weiss, 1981) noted the existence of nerve fibers surrounded by splenic cells at some distance from smooth muscle, or adjacent to lymphocytes, reticular cells, or erythrocytes. Such findings were either viewed with caution (Blue and Weiss, 1981), or dismissed out of hand as mistaken identification of some other cells such as platelets (Heusermann and Stutte, 1977). Our use of TH immunocytochemistry permits the positive identification of sympathetic nerve terminals and reveals direct contacts with lymphocytes. We consider these contacts , clearly more intimate and regular than the widely spaced association between NA terminals and smooth muscle cells, to represent synapses. Thus, the likelihood exists of both local paracrine secretion of NE into the splenic parenchyma and true NA synapses with lymphocytes. Perhaps each mode of neurotransmission subserves different functions, depending upon the distance, the concentration of NE, and the state of the target cell adrenoceptors. Since TH^+ terminals intimately contact "mobile" target cells (i.e. lymphocytes), we must re-evaluate our definitions of "innervation" in the periphery, and must overcome the bias that NA fibers in the spleen can only innervate smooth muscle cells and can therefore control only blood flow and splenic contraction. The novel forms of innervation we describe here may represent a key link between the autonomic nervous system and the immune system, as supported by our functional studies to be discussed below.

We also have examined possible cholinergic innervation of the spleen. Neurochemical analysis of acetylcholine (ACh) using high performance liquid chromatography has revealed a barely perceptible presence of this neurotransmitter, not consistent with a parasympathetically innervated organ. In addition, no choline acetyltransferase (ChAT) activity has been detected in the spleen. The acetylcholinesterase (AChE) staining in the spleen appears to be associated largely with non-neural elements, and is present after subdiaphragmatic vagotomy. If some AChE-positive profiles are associated with neural elements, that staining may be co-localized in noradrenergic nerves, as it is in gut-associated lymphoid tissue, and in many other non-lymphoid organs, and is not a reliable marker for the presence of cholinergic nerves. We therefore conclude that cholinergic innervation of the spleen is either non-existent or extremely sparse. In view of the lack of availability of the transmitter, and the presence of AChE that would probably hydrolyze any ACh that managed to reach the spleen (unlikely), it is unlikely that ACh has a physiological role in the spleen, and the lack of such fibers certainly precludes any reasonable pharmacological or surgical manipulations.

2. Lymph Node Innervation

Fluorescence histochemical and immunocytochemical observations have revealed NA innervation in specific compartments of the cervical, popliteal, and mesenteric lymph nodes (LN) in rats and mice (Williams et al., 1981; Williams and Felten, 1981; D. Felten et al., 1984; Ackerman et al., 1987). NA postganglionic sympathetic fibers entered the LN from the hilus and distributed into two major sites: the medullary cords and the capsular/subcapsular system. Nerve fibers in the medullary cords traveled with the vessels, and also branched away. These fibers continued past the cortico-medullary junction and branched into the paracortical region, avoiding the nodular or follicular zones. Some fibers continued into the outer cortical region, extending among cells of this zone. Additional NA fibers from the capsular/subcapsular system traveled into the cortical region. The cells in the paracortical and cortical regions were positive for pan-T lymphocyte markers (OX19, W3/13). Thus, several compartments of the LN received NA innervation, including the medullary cords, the paracortex and subcapsular cortex, the parafollicular areas in the cortex/paracortex, the subcapsular sinus, and the capsule.

The compartmentation of noradrenergic nerve fibers in the spleen and LN shows numerous similarities, suggesting a common functional role in both organs. Innervated sites include the major compartments listed in Table I. The diminution of primary and secondary spleen and LN PFC responses (Livnat et al., 1985) following sympathetic denervation may indicate a role of NE at sites (2) and (3). Infused catecholamines have been shown to stimulate the egress of activated lymphocytes into the circulation from the spleen (Ernstrom and Sandberg, 1973; Ernstrom and Soder, 1975) and LN (Moore, 1984). This suggests an additional functional role for NE at site (4).

3. Development of Splenic Innervation

NA innervation of the rat spleen occurs almost exclusively in the postnatal period (Ackerman et al., 1987; D. Felten et al., 1987b). At birth, only a few scattered fibers were present along the hilar vessels, a condition that persisted through 3 d. of age. By fluorescence histochemistry, these fibers appeared to follow the blood vessels into the newly forming white pulp; however, TH immunocytochemical observations have revealed these fibers to be running parallel to the central arteries, several cell layers distant from the smooth muscle cells, forming small arcing profiles. Between 3 and 7 d. of age NA fibers formed a loose network between the central artery and the developing marginal zone. This pattern of development occurred earliest in the hilar region and latest in more distal regions. By 7-10 d. of age, these fibers defined the developing marginal zone, along which $ED3^+$ macrophages clustered. Since the nerve fibers preceded the formation of the marginal zone and the clustering of macrophages at this site, they may provide a contact guidance system or trophic influence for the development of this compartment. By 14 d. of age, corresponding to the development and reorganization of the PALS, the plexus around the central artery and its branches increased in density and showed an adult pattern of both vascular and parenchymal innervation.

From 14 to 28 d. of age, the pattern of NA fiber innervation matured and kept pace with the expanding PALS and marginal zone. During d. 21 to 28, the time of follicular development, a modification of existing patterns occurred. Marginal zone fibers extended along both the inner and outer borders of the follicles forming a parafollicular network. From d. 28 to adulthood no new compartmentation or modifications of existing innervation patterns were detected, consistent with absence of further morphological change in the white pulp. The presence of NA innervation in specific compartments of the spleen during critical periods of development suggests a role for NE in the maturation of the immune system. This notion receives support from the finding that neonatal sympathectomy produces alterations in adult immune function, and may be related to the differing results of neonatal vs. adult denervation.

This developmental model provided an opportunity to examine the role lymphocytes played in the ontogeny of NA innervation. At 2-3 d. of age, rats were thymectomized (Tx) to reduce further population of the PALS with T cells. Preliminary fluorescence histochemical observation showed normal development of NA fibers through 14 d. The fibers were found in the appropriate compartments, although their density in the PALS appeared higher in the Tx rats than in sham-operated and non-operated controls, most likely due to the considerably reduced size of the PALS. By 28 d. of age, this robust innervation was reduced and fluorescent profiles were barely detectable in the central arteriolar system, the PALS, the marginal sinus, and the trabecular system. In Tx rats showing moderate to robust innervation at 28 days of age, examination at sacrifice revealed incomplete Tx. Therefore, orderly arrival of T lymphocytes into the PALS appears to be necessary for long-term maintenance, but not initial development, of the NA nerve fibers. We hypothesize that the arriving lymphocytes either release growth factors that help maintain nerve fibers, or induce the production of nerve growth factor (NGF) by other cells in the spleen. This hypothesis is consistent with recent findings in skin that NGF receptors on nerve fibers appear only after the fibers already have reached their target site (Davies et al.,

1987). NGF may be more important in the *maintenance* than the initial induction of sympathetic innervation.

4. Aging Changes in Noradrenergic Innervation of Spleen

The mature pattern of NA innervation of specific compartments of the spleen persists through approximately 18 mo. of age in F344 rats. From 18 to 27 mo. of age, the NA fibers showed progressive diminution in all compartments of spleen (S. Felten et al., 1987; Bellinger et al., 1987). At 27 mo. the total NA content was diminished by approximately 50%, and the number of fluorescent profiles, evaluated morphometrically, was reduced by approximately 80% in the parenchyma and around the central arteriolar system. However, all compartments showed at least a 60% reduction in the number of nerve profiles present on a volume percent basis. The diminished NE content could be explained in two ways: (1) the terminals were present but were unable to synthesize enough NE to form observable fluorophore or enough TH for immuostaining; or (2) the terminals were absent. To resolve this issue, we administered alpha-methylnorepinephrine, a compound that is taken up by the high affinity carrier into NA terminals and persists because it cannot be catabolized by monoamine oxidase . Alpha methylnorepinephrine was able to restore fluorescence in a few profiles in 27 mo. old rats compared with untreated controls, but not to the level of 4-18 mo. old rats. We interpret these findings to indicate actual retraction and loss of NA fibers in the aging spleen.

Preliminary observations in the LN of aging rats and mice have revealed denervation similar to the spleen. This is remarkable in view of the persistence of NA sympathetic innervation in other organs, such as the heart, in F344 rats aged 2 yr. or more. Indeed, measurement of circulating catecholamines suggests an *increase* with age. The decreased NA innervation was not found in the thymus. Rather, intact innervation with a greatly increased density of varicosities in the cortex, presumably related to the considerable shrinkage of this organ, was observed in 27 mo. old rats. Two possible bases for this unique age-related NA denervation of secondary lymphoid organs are: (1) primary denervation, which may contribute directly to diminished immune responses in aging; or (2) denervation secondary to altered numbers or activities of other cells in the lymphoid organs. For example, if NGF secretion or availability in spleen and LN decreased with age, retraction of NA fibers would be one predicted consequence. These testable hypotheses are currently are being explored in our laboratories.

5. Plasticity of Noradrenergic Fibers in the Spleen

The presence of abundant NA nerve fibers in T cell areas of the white pulp provided an excellent opportunity to manipulate nerve-target cell interactions using immunosuppressive drugs to deplete lymphocytes without damage to the nerves (Carlson et al., 1987b). Adult C3H/HeJ mice were treated with hydrocortisone acetate (2.5 mg/mouse) or cyclophosphamide (50 mg/kg or 250 mg/kg), which deplete both T and B lymphocytes in spleen. Within 48 hr. the spleens were significantly reduced in both weight and cellularity, but the total NE content remained constant. In the surviving PALS, which was reduced in size, the NA fibers were more densely clustered. Thus, NA innervation retains its compartmentation even in the face of significant loss of target cells, and shows a remarkable plasticity in its ability to react to the changing geometry of that compartment. This plasticity involves movement of fibers, but without apparent sprouting or retraction. In these lymphocyte-depleted spleens, the total content of NE was normal, but the concentration (per mg protein or per wet weight) was elevated significantly. This increased neurotransmitter concentration may play a role in the subsequent repopulation process, a hypothesis which will be tested by chemical sympathectomy of animals following cyclophosphamide treatment.

B. Criteria for Neurotransmission by Noradrenergic Nerves in Lymphoid Organs

The first criterion for neurotransmission is the presence and localization of nerve fibers that contain the neurotransmitter. Not only are the NA fibers present in primary and secondary lymphoid organs, but are precisely compartmentalized in functionally similar regions in the secondary organs. Immunocytochemical localization of TH provides evidence for the presence of the rate-limiting enzyme of NE biosynthesis in the same network of fibers that contain NE revealed by fluorescence histochemical, neurochemical, and denervation studies. Thus, criteria for presence and localization of NA fibers, and their capability to synthesize NE, have been fulfilled in all secondary lymphoid organs examined.

Although the NA nerve terminals certainly possess the capability to take up circulating NE, most NE in the spleen and LN is of neuronal rather than "hormonal" origin. Treatment of mice or rats with 6-hydroxydopamine (6OHDA) , a neurotoxin that destroys NA sympathetic nerve terminals without damaging the adrenal medullary catecholamine-secreting cells, results in a 95% or greater depletion of the total content of splenic or LN NE, suggesting its derivation from nerves and not from the circulation (Williams et al., 1981). However, it should be noted that other circulating monoamines can influence sympathetically innervated target tissues by uptake into intact terminals. Recent physiological studies show that NA nerve fibers can take up epinephrine using a high-affinity uptake carrier and can subsequently release this "hormone" as a classical neurotransmitter (Berecek and Brody, 1982). Serotonin (e.g. derived from platelets) also may be taken up by a low-affinity carrier into beds of NA terminals, and subsequently released (Cohen, 1985). Thus, while NE is the predominant neurotransmitter in NA sympathetic fibers, it is not the only transmitter available. Not only externally-derived epinephrine and serotonin, but also locally synthesized neuropeptides, may be co-localized with the NE, as occurs frequently in other sites in the central and peripheral nervous systems. The co-localized molecules may be co-released with NE, may modulate NE, or may be modulated by it. We recently have found neuropeptide-Y and substance P immunoreactivity in nerve fiber profiles in the splenic white pulp. The neuropeptide-Y profiles show a remarkable similarity in distribution to the tyrosine hydroxylase profiles, raising the possibility that neuropeptide Y may be co-localized with norepinephrine in nerves in the spleen, similar to the co-localization described in many other systems of innervation. The substance P profiles were associated with central arterioles, and also showed some distribution in the parenchyma, but with initial observations did not appear to be entirely overlapping with the noradrenergic patterns. Double labeling studies will be used to resolve this possibility of peptides co-localized with norepinephrine in splenic nerve profiles in the white pulp. It is likely, however, that some of the peptide-containing profiles are independent of the noradrenergic terminals.

These possibilities complicate interpretation of stimulation or denervation studies; such treatments may affect multiple bioactive molecules for which lymphocytes and other cells of the immune system possess receptors. It is possible that NE never occurs in the absence of other co-localized molecules, and must therefore be considered in the context of other neurotransmitters, other hormones, and cytokines that are present in the microenvironment . This raises a further caution regarding pharmacological and in vitro studies using catecholamines and their antagonists. NE, presented out of context of its normal milieu of multiple paracrine substances, may have different effects depending upon other molecules simultaneously available. This view begins to blur the distinctions between classical hormones (of the endocrine, nervous, and immune systems), and local paracrine secretions of cytokines and neurotransmitters. Recognition of this complexity has focused our attention on the specific structural relationships between chemically-identified nerve terminals and their target cells in vivo; it also underscores the importance of understanding release and availability of the neurotransmitter, as well as presence and dynamics of adrenoceptors on specific subsets of target cells.

The second criterion for neurotransmission is release. Early studies of catecholamines employed the spleen as a rich source of both the neurotransmitter and its

associated enzymes, and found that stimulation of the splenic nerve (almost exclusively NA postganglionic sympathetic fibers) released NE (von Euler, 1946). We have utilized in vivo dialysis techniques, first developed for monitoring of brain neurotransmitters, to study the spleen (S. Felten et al., 1986). This technique permits the equilibration of small molecules such as catecholamines and their metabolites from the extracellular fluid into fluid pumped through dialysis tubing implanted in the tissue. Using a microdialysis system anchored in a rat spleen, a concentration of NE of approximately 1 μM can be measured under baseline conditions. This is further evidence of NE release from an almost exclusively neural compartment. This NE concentration is considerably higher than in blood, and suggests local neural release and subsequent diffusion through the spleen, consistent with a paracrine mechanism. Superimposed on this is the presence of the direct contacts between nerve fibers and lymphocytes, described above. These synaptic contacts may provide the transmitter in even higher concentration for a more immediate effect than is available at a distance. Thus, the microenvironment may contain varying concentrations of NE depending upon the proximity of, or direct contact with, nerve terminals.

The third criterion for neurotransmission is the presence of appropriate receptors for the neurotransmitter on target cells Adrenoceptors have been described on lymphocytes, macrophages, and neutrophils. Their functional role in immune responses will be discussed in greater detail below. Beta adrenoceptors are linked with adenylate cyclase and cyclic AMP generation as a second messenger; therefore NE feeds into a signal transduction pathway stimulated by a host of other bioactive molecules, and known to be involved in immunoregulation.

The fourth criterion for neurotransmission is a functional role for the innervation, involving the specific neurotransmitter, that can be demonstrated by pharmacological or physiological manipulation. We have addressed this issue extensively, as discussed in the next section.

C. Immunologic Studies of the Role of Norepinephrine in Immunomodulation

The presence of NA fibers in primary and secondary lymphoid organs, coupled with evidence for release of NE at these sites, argues strongly for a physiological role of the sympathetic nervous system in immunological processes. Much of our work has been directed toward understanding the role of sympathetic innervation in the regulation of immune responsiveness in adult mice.

Very little is known about the neural regulation of processes occurring in the central lymphoid organs. Sympathetic innervation of the thymus and bone marrow may subserve a function in the generation, maturation, or emigration of developing lymphocytes. The work of Singh and colleagues (Singh, 1979, 1984, 1985; Singh and Owen, 1976; Singh et al., 1979) has provided evidence for sensitivity of developing thymocytes to catecholamines, in both proliferative activity and expression of surface antigens. Sympathetic innervation was first evident at the 17th day of gestation in the BALB/c mouse, though sensitivity of fetal thymocytes to adrenergic agonists was seen even earlier (d. 14). The developing thymus appeared to be under inhibitory sympathetic influence. In fact, fetal non-lymphoid thymic rudiments from nude mice supported the development of thymocytes when transplanted into the anterior chamber of a surgically sympathectomized eye. We have obtained preliminary evidence that thymocytes from 10 d. old rats sympathectomized at birth (by s.c. injection of 6OHDA), showed enhanced proliferative responses in vitro upon incubation with IL2 containing supernatants (Livnat et al., unpublished).

Even less is known about sympathetic neural effects in the bone marrow. Webber et al. (1970) reported that stimulation of the sympathetic trunk caused a release of blood cells (reticulocytes) from the marrow into the circulation. Byron (1972) found that stimulation of beta adrenoceptors triggered stem cells into cycle (or shortened their cycle). Collectively, these few observations on central lymphoid compartments suggest that cell

proliferation, differentiation, and emigration may each be subject to the regulatory influence of the sympathetic nervous system.

Our recent approach to assessing the impact of NA innervation on the immune system has been the examination of immune function following removal of that innervation by chemical sympathectomy of adult mice. Several previous studies reported varying effects of sympathetic denervation. Surgical sympathectomy of the spleen in rats (Besedovsky et al., 1979) was found to enhance the antibody response (splenic PFC). Unilateral sympathetic ganglionectomy of the superior cervical ganglion in mice 7 d. prior to regional immunization led to enhanced PFC responses in ipsilateral cervical LN (Alito et al., 1985) and augmented delayed hypersensitivity (DH) responses in the ipsilateral ear (Braun et al., 1986).

Chemical sympathectomy with 6OHDA was originally found to cause long-lasting destruction of peripheral NA fibers , accompanied by reduced NE levels, when the agent was administered over several days to neonatal animals (Jonsson et al., 1979). When rats or mice were treated in this manner, allowed to mature, and then immunized as adults, their antibody responses were also found to be augmented (Besedovsky et al., 1979), although adrenalectomy was often required to see these effects. Presumably this abolished any compensatory catecholamine synthesis by the adrenal medulla (Mueller et al., 1969). Initial studies from our laboratories utilizing such neonatal sympathectomy followed by treatment of the adults with 6OHDA (Williams et al., 1981), also revealed increased PFC responses in the spleen, which were potentiated further by pharmacologic inhibition of adrenal catecholamine synthesis. These various findings were consistent with earlier observations (discussed below) that adrenergic stimulation in vitro depressed lymphocyte proliferation, and led to the notion that the immune system is constitutively suppressed by the sympathetic nervous system.

In contrast, chemical sympathectomy of *adult* mice, followed closely by immunization, resulted in reduced (Kasahara et al., 1977; Hall et al., 1982; Felten et al., 1984 ; Livnat et al., 1985) or unaltered (Miles et al., 1981) antibody responses to T-dependent antigens. Enhanced responses to a thymus-independent antigen was reported by Miles et al. (1981). Our detailed analysis of antibody responses to SRBC in the spleen after i.p. immunization, or in popliteal LN after regional s.c. immunization, in several inbred strains of mice, showed that: (1) primary PFC responses were suppressed in all 4 strains of mice examined (40 - 97% reduction); (2) the dose of 6OHDA required for significant immunosuppression differed among strains, and appeared to be related to the degree of destruction of NA fibers in lymphoid tissue; C3H/HeJ and BALB/cByJ were more sensitive than C57BL/6J and DBA/2J mice; (3) LN responses were more sensitive than splenic responses; (4) secondary PFC responses in both spleen and LN were reduced when sympathectomy was performed prior to secondary challenge in primed mice; (5) suppressed responses were not due to adrenergic actions of NE released by dying nerve fibers, since treatment with propranolol at the time of denervation did not alter the effect ; (6) 6OHDA , although toxic to lymphocytes in vitro at high concentrations (> 0.1 mM), was not acting directly on the lymphoid cells in vivo.

More recently, we have examined the impact of sympathectomy on the development of cell-mediated immune responses in vivo (Madden et al., 1986; Livnat et al., 1987 a,b) In one approach, mice were given 6OHDA 1 and 3 d. before epicutaneous sensitization with trinitrochlorobenzene, and were ear challenged 5 d. later. Significant reduction of the ear swelling response was observed. However, marked inhibition of the response was also seen when 6OHDA was administered to sensitized mice, even 1 d. before challenge. Important pharmacologic control studies using the NA uptake blocker , desipramine, showed that 6OHDA had to be taken up into nerve terminals for the suppression to occur. These findings again argue strongly against a direct effect of 6OHDA on lymphoid cells. The fact that the beta blocker propranolol did not alter the 6OHDA-induced changes also argues against the possibility that catecholamines released

from dying nerve terminals (or 6OHDA itself), acting on beta adrenoceptors, were responsible for the immunological changes.

We do not yet know whether sympathetic denervation prior to sensitization independently influenced the inductive phase of the response or whether residual effects of the treatment altered events occurring at the time of challenge several days later. Experiments designed to study in vivo events in the inductive phase measured DNA synthesis in draining LN using the uptake of ^{125}I-iododeoxyuridine. Results of several such experiments are summarized in Table II. Contrary to our expectations, we observed that 6OHDA treatment enhanced the uptake in LN responding to suboptimal doses of antigen, and modestly inhibited the response to a "plateau" dose. In fact, LN from non-immune mice showed stimulation of DNA synthesis following 1 or 2 injections of 6OHDA. Although we do not yet know the mechanisms responsible for these results (e.g. activation of suppressor T cells, inappropriate compensatory lymphocyte proliferation) it is clear that the "afferent" phase of the immune response is modified by sympathetic denervation.

Draining LN cells from mice given 6OHDA and then primed with trinitrochlorobenzene showed reduced in vitro responses to stimulation with trinitrophenyl (TNP) hapten-modified syngeneic spleen cells. This was manifest as an approximately 50% decrease in IL2 production and in the generation of TNP-specific cytotoxic T lymphocytes (CTL) (Madden et al., 1987; Livnat et al., 1987 a,b). The observed suppression of the efferent phase of the DH response may be due, at least in part, to a deficit in the lymphokine-producing capacity of T cells. In addition, the migratory properties of sensitized T cells may be different in an altered neural environment. This possibility is supported by our preliminary observation of altered LN cell migration patterns in sympathectomized mice (Livnat, unpublished). ^{51}Cr-labelled LN cells from 6OHDA-treated C3H mice showed markedly decreased localization (1 hr. after i.v. infusion) in LN of control recipients whereas localization to the spleen and liver was largely unaffected. In contrast, 1 hr. migration of normal LN cells into sympathectomized recipient LN was greatly enhanced. We are currently examining later time points (e.g. 24 hr. migration), as well as the migration of *sensitized* LN cells. We must also consider the possibility that cutaneous components of the contact hypersensitivity response, such as antigen processing or presentation by Langerhans cells, may have been altered. Clearly, dissection of these alternatives awaits results of further studies, including adoptive transfer of cells between control and sympathectomized animals.

Recent work by Braun and her colleagues (Alito et al., 1985; Braun et al., 1986) provides additional evidence for sympathetic effects on the efferent phase of the DH response. Unilateral sympathetic ganglionectomy of immune mice modified (either stimulated or inhibited, depending on the timing) the ear swelling response to epicutaneous challenge only on the denervated side. Together with our results, these findings are evidence for a potential role for neural NE at sites 1-4 outlined in Table I.

The suppression of cell-mediated immunity by adult sympathectomy is not limited to the contact sensitivity model. We have also found (Livnat, 1986; Livnat et al., 1987 a,b) that 2 injections of 6OHDA, 150 mg/kg, 1 and 3 days before s.c. immunization with allogeneic spleen cells caused up to 50% reduction in the development of CTL in the draining popliteal LN. A similar effect was observed in vitro; 6OHDA treatment of non-immune C3H mice resulted in about 50 % inhibition of the spleen and LN T cell proliferative response in mixed lymphocyte culture and a similar reduction in the generation of allospecific CTL. However, if mice were denervated after alloimmunization, the secondary CTL response of immune LN cells was not diminished. This is in contrast to the reduction in a hapten-specific secondary CTL response, which could be overcome by the addition of T cell growth factors in vitro. These results suggest that T helper cells may be particularly sensitive to the loss of NA innervation.

Adult sympathectomy with 6OHDA does not appear to be uniformly, and non-selectively, inhibitory to the immune system. We have found, for example, (Madden et al., 1986; Livnat et al., 1987b) that the activity of natural killer (NK) cells was stimulated by prior sympathectomy. Both baseline and poly I:C-stimulated cytotoxic activities of non-immune spleen cells were increased several-fold by prior treatment of mice with 6OHDA. Similarly, in vivo NK activity in the lungs, measured as the ability to reject radiolabelled tumor cells infused i.v., was potentiated by sympathetic denervation. Consistent with this, we have observed (Eisen et al., 1987; Livnat et al., 1987b) that enhancing the presence of NE by chronic treatment of mice with the NA reuptake blocker, maprotiline, inhibited NK activity in vitro and in vivo. In vitro experiments with this and other reuptake blockers indicated that direct toxic effects on NK cells were not a likely basis for our findings. However, since these agents also act in the CNS, a centrally-mediated effect cannot be ruled out. Chronic infusion of NE into mice was expected to have an effect similar to reuptake blockade. To our surprise, we found that several doses of NE administered via s.c. implanted pellets (~0.5 to 5 µg/kg/day) for 7 d. resulted in enhanced NK activity (Livnat et al., 1987b) This result, apparently mimicking denervation, may be explained by the recent finding that NE infusions can actually induce sympathectomy (I. Azevedo, pers. comm.).

In order to understand the cellular mechanisms by which loss of sympathetic neurons leads to suppression of T cell responsiveness in vivo, we began to examine the direct effects of NE and other more selective adrenoceptor agonists (and antagonists) on CTL responses in vitro. Adrenergic agents were added on d. 0 to mixed lymphocyte cultures of C3H responder T cells (unfractionated spleen and LN cells) stimulated by irradiated BALB/c spleen stimulator cells. CTL activity was assessed in a 4 hr. ^{51}Cr-release assay 5 d. later, using P815 target cells. NE and epinephrine, in the nM to µM range, significantly potentiated the CTL response. The results of several experiments are summarized in Table II. The cellular targets of this adrenergic effect is not yet known. Viable cell yields, and incorporation of ^{3}H-thymidine were often unaltered or only modestly increased in cultures in which CTL activity was significantly enhanced, suggesting that CTL precursors are a key target of the agonist.

The adrenoceptor selectivity of the potentiated CTL response was tested using a variety of more selective agonists (Table II). Whereas the beta agonist isoproterenol was stimulatory, the $beta_2$-selective agonist,terbutaline, caused larger, more consistent augmentation of the CTL response. The $beta_1$-selective agonist dobutamine had no effect. The $alpha_1$ agonist, methoxamine,was stimulatory in some experiments, whereas the $alpha_2$ agonist, clonidine, showed stimulation and inhibition, with a peculiar dose-response relationship.

Adrenoceptor blockers were used as an additional tool for analysis. The potentiating effects of mixed agonists (NE and epinephrine) were only partially inhibited by the beta blocker, propranolol. The non-specific local anesthetic effects of this compound which itself could suppress CTL responses, precluded its use at sufficiently high concentrations. When an alternate beta blocker, timolol, with much lower membrane-paralyzing activity was tested. , virtually complete reversal of the agonistic effect was obtained (see Table III) The alpha blocker phentolamine also caused partial inhibition of the agonist response, and in the case of NE, was additive with propranolol. These results do not allow firm conclusions regarding the relative role of alpha adrenoceptors, although there is very little direct evidence for the presence of alpha receptors on cells of the immune system.

Our results do support the notion of a $beta_2$ receptor - mediated stimulation of the CTL response in vitro, and are consistent with the detection of $beta_2$ receptors on lymphocytes by radioligand binding (Brodde et al., 1981; Landmann et al., 1981,1984). Similar effects on CTL responses were reported by Hatfield et al. (1986); in addition, they provided evidence for a possible alpha receptor mediated inhibition of the CTL

response, expressed as an enhancement of the NE effect in the presence of an alpha blocker. The findings of Sanders and Munson and their colleagues (Sanders and Munson, 1984a, 1984b, 1985a, 1985b; Sanders et al., 1985), studying in vitro primary antibody responses of mouse spleen cells, are analogous in many ways. They reported that NE (10 μM) produced a 2-4-fold enhancement of the IgM PFC response, an effect which was blocked by propranolol (beta blocker) but not phentolamine (alpha blocker). Further analysis showed that $beta_2$-stimulation with terbutaline mimicked the NE effects over a wide concentration range, and that effects of both NE and terbutaline were blocked by a selective $beta_2$ blocker. Stimulation of the $alpha_1$ receptor with methoxamine produced an earlier peak in the PFC response (by 1 d.) but did not change its magnitude; similar results were obtained with the mixed agonist NE in the presence of propranolol to prevent the beta receptor-mediated potentiation. The $alpha_2$ agonist clonidine reduced the d. 5 response by 50% but had no effect on PFC number earlier or later.

Sanders et al. (1985) observed that exposure to the agonist during the initial 6 hr. of culture was sufficient to evoke maximal stimulation measured 5 d. later. Our experience with the kinetics of potentiation of the CTL response has been different. We found that delayed addition of the agonist (NE or terbutaline), on d. 1 or d. 2 of a 4 d. culture caused greater potentiation than addition at the start. This effect occurred with both non-immune and alloantigen-primed lymphocytes. Based on this finding we hypothesize that beta adrenoceptors on T cells may be up-regulated by events occurring in the first days following antigenic stimulation, rendering the cells more sensitive to signals delivered to these receptors. Studies are in progress examining the expression of beta receptors on lymphocytes at various times after antigenic (or mitogenic) stimulation in vitro or in vivo. Our preliminary results indicate that lymphocyte beta receptors show dynamic changes following immune stimulation. In LN cells draining the site of epicutaneous immunization with a contact sensitizing agent, the number of beta receptors, detected by binding of the radioligand ^{125}I-iodocyanopindolol to membrane preparations, was found to double by 4 d. after immunization. One day after immunization, we have observed increases, decreases or no change. In all cases, the dissociation constant was unchanged (Livnat et al., 1987b). Studies using whole cells and either hydrophilic ligands or appropriate washing conditions, to detect cell-surface receptors only, are currently in progress.

It is important to emphasize that cells other than T or B lymphocytes may be targets for NE action. Several macrophage functions, such as synthesis of complement components (Lappin and Whaley, 1982) and activation to a cytocidal state (Koff an Dunegan, 1985), were inhibited by NE or alpha agonists. Antigen processing or presentation, and IL1 synthesis or secretion could also be affected. This adds a great deal of complexity to the earlier notions, derived from studies of mitogen responses (Hadden et al., 1970; Johnson et al., 1981) or antibody secretion (Melmon et al., 1974), which stated that beta-adrenergic stimulation, by elevating cAMP levels, inhibited lymphocyte proliferative and secretory functions, and thus served as a simple negative regulatory signal. Elevation of cyclic GMP by stimulation of muscarinic cholinergic receptors (presumably the mode of action of the parasympathetic nervous system), enhanced lymphocyte proliferation in vitro (Strom et al., 1977, 1981; Gordon et al., 1978). These observations were unified into a sort of "Yin-Yang" theory of immunoregulation (Hadden,1977) which viewed cAMP-elevating stimuli as suppressing, and cGMP-elevating stimuli as potentiating, immune responses.

Such a simple "linear" notion appears untenable today. Elevation of cAMP may promote cellular differentiation as it blocks cell growth. Since an orchestrated immune response requires a complex combination of cells undergoing activation, proliferation, differentiation, and cessation of these activities in a timely fashion, it is impossible to equate inhibition of cell proliferation to "immunosuppression". Much has been learned about pathways of signal transduction in lymphocytes in recent years (see Immunological Reviews 95: 1987). Different adrenergic (and cholinergic) receptors in various tissues are

coupled to various pathways which: (1) activate or (2) inhibit adenylate cyclase and cAMP generation; (3) activate guanylate cyclase, generating cGMP; (4) stimulate inositol phosphate turnover and elevate intracellular free calcium. The matrix of possible ligand-receptor-transduction combinations which may exist in a single T lymphocyte, for example, is daunting. When one superimposes additional immunophysiological mechanisms not readily studied in cell culture, such as antigen localization and retention, lymphocyte migration and recruitment, one cannot help but be struck by the host of sites which may be subject to coarser or finer levels of neural regulation.

Herein lies both the frustration and the challenge of such studies, in which both the interactions of complex systems and the detailed mechanistic knowledge of neurotransmitter-receptor interactions are needed. We need to understand the dynamics of adrenoceptor expression on the various sets and subsets of immune cells, and then bring it to the level of the architecturally intact microenvironment, to permit integration with information about the localization of nerve fibers, and the release, and available concentrations, of neurotransmitter.

III. PUBLICATIONS (Year 1 of renewal)

Manuscripts

1. Felten, S.Y., S.L. Carlson, D.L. Bellinger, and D.L. Felten 1986 Overview of the efferent autonomic nervous system. In: R. Frederickson, H.C. Hendrie, J.N. Hingtgen, M.H. Aprison (Eds.), Neuroregulation of Autonomic, Endocrine and Immune Systems, Martinus-Nijhof, pp. 109-126.

2. Ackerman, K.D., S.Y. Felten, D.L. Bellinger, S. Livnat, and D.L. Felten 1987 Noradrenergic sympathetic innervation of spleen and lymph nodes in relation to specific cellular compartments. Prog. Immunol. 6:588-600.

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TABLE I

INNERVATED COMPARTMENTS OF SECONDARY LYMPHOID ORGANS

SITE OF:		SPLEEN	LYMPH NODE
1. LYMPHOCYTE ENTRY		MARGINAL ZONE	CORTICOMEDULLARY JUNCTION
2. ANTIGEN CAPTURE		MARGINAL ZONE	SUBCAPSULAR SINUS
3. ANTIGEN PRESENTATION / LYMPHOCYTE ACTIVATION	T:	PALS	PARACORTEX
	B:	PARAFOLLICULAR / MARGINAL ZONE	MEDULLARY CORDS
4. LYMPHOCYTE EGRESS		OUTER MARGINAL ZONE	MEDULLARY SINUSES

TABLE II.

SYMPATHECTOMY ALTERS ^{125}IUdR UPTAKE INTO DRAINING LYMPH NODES FOLLOWING EPICUTANEOUS IMMUNIZATION OF BALB/c MICE

	DRUG TREATMENT SCHEDULE					
	Vehicle		6-OHDA (150)* (d. -1, -3)		6-OHDA (100) (d. -1)	
Immunization	n	cpm#	n	cpm	n	cpm
None	4	222 ± 35	--	---	3	1525 ± 271
Vehicle	8	384 ± 63	2	799 ± 53	6	1924 ± 221
0.2 mg TNCB†	10	1814 ± 149	4	3202 ± 358	5	3544 ± 337
2.0 mg TNCB	7	4537 ± 385	5	3071 ± 485	4	3361 ± 369

* 6-OHDA dose: 150 or 100 mg/kg i.p. at indicated times before immunization.

\# Mean cpm (+ s.e.m.) of ^{125}I in draining axillary and inguinal lymph nodes (n = number of mice).

† Trinitrochlorobenzene dissolved in acetone/olive oil vehicle; sensitization with 0.1 ml on shaved abdomen.

TABLE III.

ADRENERGIC STIMULATION OF CYTOTOXIC T LYMPHOCYTE RESPONSES

AGONIST	SELECTIVITY	EFFECT ON CTL RESPONSE	
Norepinephrine	alpha > beta	INCREASE	(40-250%)
Epinephrine	beta > alpha	INCREASE	(45-350%)
Isoproterenol	beta	INCREASE	(25-120%)
Dobutamine	$beta_1$	NO CHANGE	
Terbutaline	$beta_2$	INCREASE	(50-500%)
Methoxamine	alpha	± INCREASE	(0- 80%)

Adrenergic compounds in the nM to μM range were added to mixed lymphocyte cultures (C3H spleen + LN responder cells, irradiated BALB/c stimulators) in 24 well plates or upright 25cm^2 flasks. After 4-5 d. of culture, cells were harvested and CTL activity was assessed against P815 targets in a standard 4 hr. ^{51}Cr-release assay. Multiple effector:target ratios were tested and CTL activity expressed as lytic units per 10^6 cells (or per culture). Effect is expressed as % increase of lytic units compared to control cells to which diluent was added. (e.g. 50% increase = 150% of control). Results are based on 3-11 experiments with each compound.

TABLE IV.

INHIBITION OF POTENTIATED CTL RESPONSES BY ADRENOCEPTOR BLOCKERS

AGONIST (0.1 μM)	BLOCKER (0.1 μM)	REDUCTION OF AGONIST RESPONSE (%)
Norepinephrine	Propranolol [beta]	42 - 45
	Phentolamine [alpha]	25 - 31
	Both	75 - 85
Epinephrine	Propranolol	30 - 40
	Phentolamine	25 - 31
	Both	36 - 47
	Timolol [beta] (1μM)	82
Terbutaline	Propranolol	48 - 54
	Timolol (1μM)	94

Experiments were performed as described in Table III., with the blockers added at the initiation of culture. Results are expressed as % Reduction of the agonist-induced potentiation of the CTL response. Thus, 100% reduction indicates that the response was suppressed to background levels (with no agonist added). Ranges of values indicate results of 2-4 experiments.

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